(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification⁷: **A61K**

(21) International Application Number: PCT/US02/12251

(22) International Filing Date: 17 April 2002 (17.04.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/287,426   30 April 2001 (30.04.2001)   US
10/094,365   8 March 2002 (08.03.2002)   US

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(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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(54) Title: METHODS FOR INHIBITING MACROPHAGE COLONY STIMULATING FACTOR AND C-FMS-DEPENDENT CELL SIGNALING

(57) Abstract: Described herein are methods of inhibiting M-CSF activity, and, in particular, M-CSF/c-fms dependent cell signaling. In a first embodiment of the invention, one administraters to a mammal viral vectors that deliver genes expressing antisense *c-fms* RNA; in a second embodiment, one induces *in vivo* production of a high-affinity soluble *c-fms* protein that competes for non-bound M-CSF; in a third embodiment, one administers a ribozyme-viral vector against *c-fms* mRNA; and in a fourth embodiment, one administers oligodeoxynucleotides that inhibit expression of *c-fms* gene product. The methods may be used to treat any disease in which M-CSF activity plays a role, and are particularly effective in treating and preventing atherosclerosis. Embodiments of the present invention are directed primarly, but not exclusively, to a method for treating and preventing cardiovascular disease by inhibiting receptors to M-CSF. Other embodiments of the present invention include any and all biologic and/or pathobiologic phenomena mediated in whole or in part by M-CSF singaling through its receptor. Pathobiologic phenomena inlcude, but are not limited to, disease entities such as osteoprosis, Alzheimer's disease, diabetes mellitus (Type 1 and/or Type 2), infectious disease, cancer, and inherited disorders characterized by defects in one or more components in the M-CSF signaling pathway.

METHODS FOR INHIBITING MACROPHAGE COLONY STIMULATING FACTOR AND c-FMS-DEPENDENT CELL SIGNALING

FIELD OF THE INVENTION

Embodiments of the present invention are directed to inhibiting macrophage-colony stimulating factor (M-CSF) cell signaling – and, in particular, the diseases caused by such signaling – by inhibiting or otherwise interfering with the expression and/or biological activity of the M-CSF receptor, the product of the proto-oncogene *c-fms*.

BACKGROUND OF THE INVENTION

Macrophage colony stimulating factor plays an important role in many biological processes, including the development of blood cells and the regulation of the immune system. It also plays an important role in atherosclerosis, osteoporosis, and other diseases. Its role in atherosclerosis is the most significant because, if anything, this disease is the leading cause of death in most developed countries.

Atherosclerosis is an inflammatory process of the arteries that begins as a fatty deposit (primarily lipid-filled macrophages), progresses to a fibrofatty lesion (all the foregoing, plus a mesh of smooth muscle cells, T-cells, collagen, proteoglycans, and elastic fibers), and ultimately leads to a fibrous plaque (all the foregoing, plus dense connective tissue and necrotic debris). If a person fails to treat the plaque or modify the diet or other lifestyle characteristics that likely contributed to it, the lesion will eventually occlude most or all of the artery, and the person will experience symptoms of heart disease such as chest pain, may have a heart attack, and might even die. Even if he escapes a heart attack, he is still vulnerable to stroke, gangrene of the extremities, damage to internal organs, and other serious diseases. As a result, atherosclerosis is the single most important cause of death and disability in developed countries in America, Europe and Asia, and will soon overtake infection as the primary cause of death in the entire world (R. Ross., "The Biology of Atherosclerosis," in E. Topol, Ed., *Comprehensive Cardiovascular Medicine*, Lippincott-Raven, Philadelphia (1998); CJL Murray, AD Lopez. Mortality by cause for eight regions of the world: Global Burden of Disease Study. Lancet. 349:1269-1276 (1997); T.A. Pearson, S.C. Smith, P. Poole-Wilson, "Cardiovascular specialty societies and the emerging global burden of cardiovascular disease: a call to action," *Circulation*. 97:602-604 (1998).

Widely accepted scientific opinion maintains that atherosclerosis is a complex inflammatory response to injury of the blood vessel, and a crucial early event in atherosclerosis is injury of some form to the arterial endothelium. Oxidized low-density lipoprotein (Ox-LDL) is a major source of injury; toxins, viruses, homocystein, and

5   mechanical injury are other sources. The interaction of these agents with the endothelium changes the endothelial cells, making them prone to further interaction with monocytes circulating in the blood. Injury to the endothelium permits monocytes and other inflammatory cell types to penetrate the outer layer of the endothelium, where they differentiate into macrophages. Macrophages proliferate within the developing lesion

10   and load themselves with Ox-LDL and acquire a foamy appearance. The lipids activate the macrophages, which then express new genes for stimulating smooth muscle cells, producing enzymes, and stimulating other macrophages, all of which make a major contribution to the development of the atherosclerotic plaque. If injury to the endothelium continues, the smooth muscle cells migrate and proliferate into the subendothelial space

15   and combine with connective tissue and other matter, thereby forming an atherosclerotic lesion. The developing lesion begins to occupy more and more volume, and eventually encroaches upon the inside of the blood vessel, thereby restricting the flow of blood, and frequently blocking the artery altogether.

The most effective known method to treat atherosclerosis is to modify, where

20   possible, the risk factors associated with it (some risk factors, such as gender, cannot be modified). Proper exercise and diet, maintaining a healthy weight, drinking in moderation, and not smoking can significantly reduce atherosclerosis and the diseases (e.g., heart attack, stroke) it causes. This is easier said than done, of course: the prevalence of atherosclerosis suggests that most individuals have difficulty making the

25   sacrifices that proper exercise and diet require or that they succumb to the disease nonetheless. The most common pharmacological treatment for atherosclerosis seeks to lower blood levels of LDL, the so-called "bad cholesterol." This method of treatment is effective in reducing the complications of atherosclerosis in many individuals, yet it is not a cure: atherosclerosis remains a leading cause of death and disability. For these

30   reasons, a basic medical text still teaches that "[t]reatment of established atherosclerosis is directed at its complications. . . ." M. H. Beers and R. Berkow, eds., *Merck Manual of Diagnosis and Therapy*, 1658 (1999).

However, if it were possible to disrupt, at the cellular level, the process that causes atherosclerosis, one could direct treatment at the disease itself, instead of its complications. For example, if there were a method to inhibit the growth, survival or activation of macrophages (or of vascular smooth muscle cells that feature prominently in the diseased artery wall), one might be able to prevent the formation and progression of atherosclerosis, and this would obviously be far preferable to merely treating its complications. The present invention is directed to such a method.

Over the years, the inventor and his colleagues have accumulated evidence which strongly indicate that M-CSF/*c-fms*-dependent cell signaling contributes to both the development and the breakdown of atherosclerotic lesions by regulating the growth, survival and function of monocyte-macrophages and intimal smooth muscle cells. T. B. Rajavashisth *et al.*, "Induction of endothelial cell expression of granulocyte and macrophage colony-stimulating factors by modified LDL," *Nature*, 344:254-257 (1990); T. B. Rajavashisth *et al.*, "Transcriptional activation of the macrophage-colony stimulating factor gene by minimally modified LDL: involvement of nuclear factor κB," *Arterioscler Thromb Vasc Biol.*, 15:1591-1598 (1995); T. B. Rajavashisth *et al.*, "Transcriptional activation of the macrophage-colony stimulating factor gene by minimally modified LDL: involvement of nuclear factor κB," *Arterioscler Thromb Vasc Biol*, 15:1591-1598 (1995); T. B. Rajavashisth *et al.*, "Atherosclerosis: from risk factors to regulatory molecules," in *Encyclopedia of Human Biology*, R. Delbacco and P. Abelson, eds. Academic Press, San Diego, California, 1:565-574 (1997). They have established this conclusion *in vivo* by studying atherogenesis in osteopetrotic *(op)* mice that lack M-CSF due to a point mutation in the M-CSF gene. Atherogenesis was induced either by feeding the mice a high fat, high cholesterol diet or by crossing *op* mice with either apolipoprotein (apo) E or LDL receptor (LDLR)- null mice to generate mice lacking both M-CSF and apo E or LDLR. J. H. Qiao *et al.*, "Role of macrophage-colony stimulating factor in atherosclerosis: studies of osteopetrotic mice," *Am J Pathol*, 150:1687-1699 (1997); T. B. Rajavashisth *et al.*, "Heterozygous osteopetrotic *(op)* mutation reduces atherosclerosis in LDL receptor deficient mice," *J Clin Invest*, 101: 2702-2710 (1998). The absence of M-CSF in both apo E or LDLR–deficient mice results in decreased atherosclerosis despite marked hypercholesterolemia suggesting that inhibition of M-CSF function in the diseased vessel wall may confer antiatherogenic properties.

An early event in the development of atherosclerotic lesion also includes migration of SMC from the media to the intima of the artery wall, where they proliferate and load themselves with lipid and thus become foam cells. J. Thyberg *et al.*, "Regulation of differentiated properties and proliferation of arterial smooth muscle cells,"

5 *Arteriosclerosis*, 10:966-990 (1990); R. Ross, "The platelet-derived growth factor," *Cell*, 46:155-169 (1986). Numerous observations suggest that during the atherogenic process SMC change their structure and function. *Id.* Although the precise mechanism of this phenotypic modulation remains unclear, several growth factors or cytokines appear to play an important role in this process. Intimal SMC derived from atherosclerotic lesions

10 express increased levels of M-CSF isoforms and its receptor, *c-fms*. R. Ross *et al.*, "Localization of PDGF-B protein in macrophages in all phases of atherogenesis," *Science*, 248:1009-1012 (1990); R. N. Salomon *et al.*, "Increased apolipoprotein E and *c-fms* gene expression without elevated interleukin 1 or 6 mRNA levels indicates selective activation of macrophage functions in advanced human atheroma," *Proc Natl Acad Sci*

15 *USA*, 89:2814-2818 (1992). SMC can express *c-fms* when activated by cytokines such as platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), heparin-binding epidermal growth factor-like growth factor (HB-EGF), or phorbol esters. T. Inaba *et al.*, "PDGF induces *c-fms* and scavenger receptor genes in vascular smooth muscle cells," *J Biol Chem.*, 267:17107-13112 (1992); T. Inaba *et al.*, "Induction of sustained

20 expression of proto-oncogene *c-fms* by platelet-derived growth factor, epidermal growth factor, and basic fibroblast growth factor, and its suppression by interferon-gamma," *J Clin Invest*, 95:1133-1139 (1995); T. Inaba *et al.*, "Synergistic effects of transforming growth factor β on the expression of *c-fms*, macrophage colony-stimulating factor receptor gene, in vascular smooth muscle cells," *FEBS Lett*, 399:207-210 (1996). These

25 results imply that activated SMC may respond mitogenically to M-CSF, a possibility with important implications for atherogenesis; that is, since M-CSF expression is increased in atherosclerotic lesions compared to the normal artery, M-CSF may play an important role in mitogenically altering SMC, and cause them to contribute to the further development of atherosclerotic lesions.

30 To examine the role of M-CSF in proliferation of arterial SMC, the inventor has previously used cultured human aortic SMC (HASMC) as a primary cell model and the injured rat carotid artery as an *in vivo* model. T. Rajavashisth *et al.*, "Role of induced

expression of M-CSF and its receptor in the growth and proliferation of intimal smooth muscle cells," *Circulation*, 88:I-468 (1993). His results indicate that induced expression of M-CSF and *c-fms* correlates strongly with the initiation of SMC proliferation observed in balloon-injured rat carotid artery. Intimal SMC express higher levels of M-CSF

5   receptor as compared to medial SMC and proliferate in response to M-CSF, thus providing indirect evidence that induced M-CSF activity not only affects monocyte-macrophage proliferation but can also affect the proliferation of intimal SMC; this suggests a broader role for M-CSF in atherosclerosis. These findings are supported by observations in osteopetrotic (*op*) mice that totally lack M-CSF. In a periadventitial

10   carotid arterial injury model in the *op/op* mouse, M-CSF deficiency markedly inhibits injury-induced intimal thickening and neointimal proliferation. Additionally, results from the effects of purified M-CSF on the growth of cultured HASMC favor the concept that intimal SMC acquire certain characteristics of monocyte-macrophages that may be related to their proliferation and phenotypic conversion into foam cells in atherosclerotic

15   lesions. Recent studies from other investigators support the inventor's finding that M-CSF can modulate the growth of vascular SMC. T. Inaba *et al.*, "Transcription factor PU.1 mediates induction of *c-fms* in vascular smooth muscle cells: A mechanism for phenotypic change to phagocytic cells," *Mol Cell Biol*, 16:2264-2273 (1996); T. Herembert *et al.*, "Control of vascular smooth-muscle cell growth by macrophage-colony-

20   stimulating factor," *Biochem J*, 325:123-128 (1997).

M-CSF is a multifunctional protein that can stimulate the growth of monocyte-macrophages, trophoblasts, osteoclasts and vascular SMC and is necessary for the survival of these cells in culture and *in vivo*. E. R. Stanley et al., "CSF-1: a mononuclear phagocyte lineage specific hemopoetic growth factor," J Cell Biochem, 21:151-159

25   (1983); R. J. Tushinski et al., "Survival of mononuclear phagocytes depends on a lineage-specific growth factor that the differentiated cell selectively destroy," *Cell*, 28:71-77 (1982). Vessel wall bound M-CSF, in particular, can regulate recruitment, cell cycle, survival and proliferation of monocyte-macrophages leading to the genesis and progression of atheromatous lesions. The ability of M-CSF to stimulate the uptake and

30   degradation of modified lipoproteins by upregulating scavenger receptors may lead to the removal of oxidized lipoproteins from the extracellular space and the generation of foam cells. S. Ishibashi, "Monocyte colony-stimulating factor enhances uptake and

degradation of acetylated low-density lipoproteins and cholesterol esterification in human monocyte-derived macrophages," *J Biol Chem*, 265:14109-14117 (1990); W. J. S. de Villiers et al., "Macrophage-colony stimulating factor selectively enhances macrophage scavenger receptor expression and function," *J Exp Med*, 80:705-709 (1994); H.

5   Shimano, "Human monocyte CSF enhances the clearance of lipoproteins containing apolipoprotein B-100 via both low-density lipoprotein receptor-dependent and - independent pathways in rabbits," *J Biol Chem*, 265:12869-12875 (1990).

        M-CSF exists in multiple isoforms. A single M-CSF gene gives rise to at least four distinct mRNAs due to alternative splicing in mice and humans. Rajavashisth et al.,

10   "Cloning and tissue-specific expression of mouse colony-stimulating factor mRNA," Proc Natl Acad Sci USA, 84:1157-1161 (1987); D. P. Cerretti et al., "Human macrophage-CSF: alternative RNA and protein processing from a single gene," *Mol Immunol*, 25:761-770 (1988). In mice, various tissues express a complex pattern of multiple M-CSF transcripts (ranging from 1.6 to 4.5 kb) in a highly tissue specific manner. These

15   transcripts encode at least two distinct proteins, secreted as glycoprotein (sM-CSF) and proteoglycan (pgM-CSF) forms, and a third membrane bound (mM-CSF) isoform with cell-surface biological activity. M. B. Ladner et al., "Human CSF-1: Gene structure and alternative splicing of mRNA precursors," EMBO J., 6:2693-2698 (1987); D. P. Cerretti et al., "Human macrophage-CSF: alternative RNA and protein processing from a single

20   gene," *Mol Immunol*, 25:761-770 (1988); E. S. Kawasaki et al., "Molecular cloning of a complementary DNA encoding human macrophage-specific colony stimulating factor (CSF-1)," *Science*, 230:291-296 (1985); S. Suzu et al., "Identification of a high molecular weight macrophage colony-stimulating factor as a glycosaminoglycan-containing species," *J Biol Chem*, 267:4345-4348 (1992); C. W. Rettenmier et al., "Synthesis of

25   membrane-bound colony-stimulating factor 1 (CSF-1) and down modulation of CSF-1 receptors in NIH3T3 cells transformed by cotransfection of the human CSF-1 and *c-fms*," *Mol Cell Biol*, 8:2378-2387 (1987). Precursors of mature M-CSF isoforms contain a common amino terminus, a spacer region of varying length, and a common trans-membrane domain at the carboxy terminus. The membrane bound precursor may be

30   processed by different proteolytic activities located at different sites in the body, producing the secreted isoform of M-CSF. It is also possible that membrane bound M-CSF molecules function as cell-associated ligands stimulating biological response in a

"juxtacrine" manner (i.e., requiring cell to cell contact). In this respect, the three different M-CSF isoforms would in effect extend the ligand binding domain of the amino terminus from the membrane, and thus allow a cell to stimulate a *c-fms* receptor on another cell located at a variable distance from the cell initiating the M-CSF signal.

5   Multiple isoforms of M-CSF utilize a single membrane bound receptor (*c-fms*), C. J. Sherr *et al.*, "Macrophage colony-stimulating factor, CSF-1, and its proto-oncogene-encoded receptor," *Cold Spring Harbor Symp Quant Biol*, 53:521-530 (1988), suggesting that this receptor may be a suitable target to inhibit M-CSF mediated cell signaling. This idea is directly supported by evidence indicating that systemic injection of a monoclonal
10   antibody against murine *c-fms* in the LDLR-deficient mice results in markedly reduced atherosclerosis. T. B. Rajavashisth *et al.*, "Monoclonal antibody against murine macrophage colony-stimulating factor receptor inhibits atherogenesis in LDL receptor-deficient mice," *Circulation* 98:A1711, 1198. These studies demonstrate that the monoclonal antibody specific to the ligand binding domain of the M-CSF receptor
15   markedly inhibits atherosclerosis in LDLR-deficient mice. Furthermore, these studies strongly support the concept that M-CSF plays a key role in the genesis and progression of atherosclerosis. Although the exact mechanism by which anti-*c-fms* antibody confers its resistance to atherosclerosis remains to be elucidated, the observation that the disruption of M-CSF/*c-fms* signaling pathways inhibits atherosclerosis suggests that
20   therapy based on disruption of M-CSF/*c-fms* signaling pathways could have potential as an effective antiatherogenic strategy.

Although anti-*c-fms* antibody inhibits atherosclerosis in mice, treating atherosclerosis (or any other disease in which inhibiting M-CSF would have a beneficial effect) by administering this antibody is not practical in humans. Producing such
25   antibodies is expensive. It demands much laboratory time and many laboratory resources. To effectively administer it, one would need to deliver frequent intravenous injections to patients, which further limits its usefulness. There is therefore a need in the art for a method of inhibiting M-CSF, and, in particular, M-CSF/*c-fms* cell signaling, that does not depend on administering anti-*c-fms* antibodies; this arises from the even
30   greater need for a method of treating atherosclerosis itself – the leading cause of death in much of the world – and not merely its complications. The inventor is the first to describe such a method, as set forth herein.

SUMMARY OF THE INVENTION

The present invention is directed to methods of disrupting the biological effects of M-CSF by inhibiting the expression and/or function of its membrane bound receptor, *c-fms*. These methods permit one to treat any disease in which inhibiting M-CSF would have a beneficial effect.

A first embodiment of the present invention is directed to a method of inhibiting M-CSF/*c-fms* cell signaling by administering to a mammal recombinant viral vectors (e.g., adenovirus, adeno-associated virus, retroviruses, lentiviruses, or other viral vectors) that deliver genes expressing antisense *c-fms* RNA; doing so inhibits the expression of *c-fms*, thereby inhibiting M-CSF/*c-fms* cell signaling. An optimal amount of viral particles and an effective and convenient route to administer it (e.g., by administering it intravenously or intramuscularly) can readily be determined by one of ordinary skill in the art of microbiology.

A second embodiment of the present invention is directed to a method of inhibiting M-CSF/*c-fms* cell signaling by inducing *in vivo* production of a high affinity soluble *c-fms* protein that competes for non-bound M-CSF but lacks all of its signal transduction domain. The method involves delivering viral vectors to produce an amount of soluble *c-fms* (or its derivative) that is sufficient to reduce the amount of M-CSF ligand and thereby inhibit the M-CSF/*c-fms* cell signaling pathway.

A third embodiment of the present invention is directed to a method of inhibiting M-CSF/*c-fms* cell signaling with somatic-cell gene therapy. According to this method, one administers a ribozyme-viral (adeno, adeno-associated, lentiviral or other) vector against *c-fms* mRNA in a mammal. The method utilizes a hammerhead ribozyme expression cassette in a viral backbone that targets the GUC sequence in codon 18 of *c-fms* mRNA. Ribozymes have sequence-specific endoribonuclease activity, which makes them useful for sequence-specific cleavage of mRNAs and further inhibition of gene expression. Ribozyme therapy is widely regarded as a new and potential pharmaceutical class of reagent to treat a number of medical disorders. A desired quantity or the length of expression of the ribozyme-viral vector can be readily determined without undue experimentation, as can the most effective and convenient route of administering it. Ribozyme-viral vectors against *c-fms* mRNA permit one to uniquely assess the

contribution of M-CSF mediated cell signaling to vascular physiology, and to therapeutically intervene in the pathology such signaling causes.

A fourth embodiment of the present invention provides a non-viral method to inhibit the expression of *c-fms*. This method involves antisense therapy using oligodeoxynucleotides (ODN) that inhibit the expression of the *c-fms* gene product by specific base pairing of single stranded regions of the *c-fms* mRNA. The method involves synthesis of ODN complememtary to the first 15 nucleotides of *c-fms* mRNA starting with the initiation codon AUG; this codon is optimized for uptake and stability by cultured mouse bone-marrow derived macrophages and vascular smooth muscle cells. The method further provides an effective amount of ODN to inhibit the M-CSF/*c-fms* dependent cell signaling pathways in a mammal.

BRIEF DESCRIPTION OF THE FIGURES

The file of this patent contains at least one drawing executed in color. Copies of this patent with color drawing(s) will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

Fig. 1 illustrates a strategy according to one embodiment of the invention to generate *c-fms* sequences expressing either antisense RNA or soluble protein according to the present invention. The upper line of the diagram shows the predicted entire protein structure of the murine *c-fms* receptor including the extracellular, transmembrane (TM) and cytoplasmic domains. The individual immunoglobulin-like subdomains and intrasubdomain disulfide bonds (s-s) are shown. The *c-fms* cDNA with the ATG start codon and the TGA stop codon is shown. The region marked with dotted lines indicates the region that encodes the ligand (M-CSF) binding domain. The cDNA encoding soluble *c-fms* is amplified using primers 1 and 2 as shown. The amino acid number contained in the soluble segment is shown. Proteolytic cleavage of pro-*c-fms* would remove the N-terminal 19 amino acids.

Fig. 2 illustrates a map of an adenoviral (Ad5) transfer vector used in a preferred embodiment of the invention and the PCR genotyping for the recombinant Ad5 expressing sense and antisense *c-fms* sequences.

Fig. 3 shows a RT-PCR assay for *c-fms* antisense RNA.

Fig. 4 shows a Northern blot analysis of *c-fms* antisense RNA.

Figs. 5A-5C (color) show that infection of cultured macrophages with Ad5CMV*c-fms*AS inhibits the growth of macrophages in a time-dependent manner (A is at 0 hours; B is at 48 hours; C depicts detection of green fluorescence protein (GFP) at 48 hours).

Figs. 6A and 6B illustrate that infection with Ad5CMV*c-fms*AS inhibits cell survival in cultured macrophage (Mφ) in a dose-dependent manner.

Figs. 7A-7F (color) show infection of mouse vascular SMC with Ad5CMV*c-fms*AS (A and D are at 0 hours, D depicts a staining for α-actin; B and E are at 48 hours; C and F are at 72 hours, F depicts detection of GFP).

Figs. 8A-8F (color) show infection of human coronary artery SMC with Ad5CMV*c-fms*AS (A is at 0 hours; B, C, D, and E depict detection of GFP at 24 hours, 48 hours, 72 hours, and 96 hours, respectively; F depicts TUNEL staining at 48 hours).

Fig. 9 shows tissue expression of antisense *c-fms* RNA following *in vivo* gene transfer of Ad5CMV*c-fms*AS particles.

Figs. 10A and 10B (color) show the effects of *c-fms* antisense RNA on atherosclerosis in apo E-deficient mice (A depicts a Ad5CMVpolyA treated mouse; B depicts an Ad5CMV*c-fms*AS treated mouse).

Fig. 11 shows that antisense *c-fms* RNA markedly inhibits atherosclerosis in apo E-deficient mice.

Figs. 12A-12C (color) show that antisense *c-fms* RNA markedly inhibits atherosclerosis in LDLR-deficient mice (A depicts a PBS treated mouse; B depicts an Ad5CMVpolyA treated mouse; C depicts an Ad5CMV*c-fms*AS treated mouse).

Figs. 13A and 13B (color) show high levels of expression of a reporter gene following perivascular delivery of the recombinant Ad5 viral vectors (A is gel only; B is gel + 10⁹ pfu/ml Ad5CMV*c-fms*AS).

Figs. 14A and 14B (color) are photographs of porcine coronary artery cross sections intramurally injected either with (A) a vector control (Ad5CMVpolyA), or with (B) a recombinant vector expressing antisense *c-fms* RNA using the INFILTRATOR®. The INFILTRATOR® is an over-the-wire balloon catheter with 3 lumens; one central lumen for the guidewire, one for balloon inflation, and another for drug infusion. This figure shows exuberant neointimal response in coronary arteries that received control viral vectors and shows appreciable inhibition in the coronary that was injected with viral vectors expressing antisense *c-fms* RNA.

Fig. 15 illustrates a scheme according to one embodiment of the invention to generate hammerhead ribozyme against codon 18 of the *c-fms* mRNA.

Fig 16 A and B shows that *c-fms* ribozyme (*c-fms*Rz) reduces *c-fms* mRNA and protein levels in cultured mouse macrophage (RAW264.7) cells.

Fig 17 illustrates two ODN corresponding to the first 15 nucleotides of either sense or antisense sequence of the human *c-fms* mRNA, starting with the initiation codon (AUG). The human *c-fms* mRNA with the ATG start codon and the TGA stop codon is shown on top.

DETAILED DESCRIPTION OF THE INVENTION

Methods of the present invention utilize RNA and protein-based inhibitors to inhibit M-CSF activity by interfering with the production and/or biological activity of a native *c-fms* receptor. One can use these methods to treat any disease in which inhibiting M-CSF activity has a beneficial effect on a patient (ameliorating a disease, lessening the severity of its complications, preventing it from recurring, or merely preventing it from worsening are examples of beneficial effects). Many diseases are known in the art in which M-CSF activity is known or suspected to play a role in initiating, aggravating, or maintaining the pathological state that comprises the disease. Atherosclerosis, osteoporosis, arthritis, Alzheimer's, nephritis (glomerular, IgA, and lupus), and diabetes mellitus (type 1 and/or type 2), infectious diseases, cancer, inflammatory diseases and inherited disorders characterized by defects in one or more components in the M-CSF signaling pathway.

Embodiments of the present invention are directed primarily, but not exclusively, to a method for treating and preventing cardiovascular disease by inhibiting receptors to M-CSF. Other embodiments of the present invention include any and all biologic and/or pathobiologic phenomena mediated in whole or in part by M-CSF signaling through its receptor. Pathobiologic phenomena include, but are not limited to, disease entities such as osteoporosis, Alzheimer's disease, diabetes mellitus (Type 1 and/or Type 2), infectious diseases, cancer, and inherited disorders characterized by defects in one or more components in the M-CSF signaling pathway. Methods of the present invention may also be used to treat those diseases in which *c-fms* is abnormally expressed and/or abnormally functions.

In a preferred embodiment, methods of the invention are used to inhibit atherosclerosis, and to thereby treat the cardiovascular diseases that atherosclerosis causes. These methods may be used in any patient who could benefit from reducing atherosclerosis that is already present, from inhibiting atherosclerosis that has yet to

5   form, or from both reducing existing atherosclerosis and inhibiting new atherosclerosis. Such patients include those suffering from, for example, angina pectoris and its subtypes (e.g., unstable angina and variant angina); ischemias affecting organs such as the brain, heart, bone, and intestines, and conditions associated with these ischemias, such as stroke, transient ischemic attacks, heart attack, osteonecrosis, colitis, poor kidney

10   function, and congestive heart failure; poor blood circulation to the extremities and the complications of poor blood circulation, such as slow wound healing, infections, and claudication; atherosclerosis itself, including restenosis following angioplasty of atherosclerotic lesions; and other diseases caused by or associated with atherosclerosis.

      M-CSF/c-fms dependent cell signaling can be disrupted in several ways according

15   to methods of the invention: one can, for example, follow a nucleic-acid based approach that inhibits or suppresses the expression of c-fms RNA; or one can follow a protein-based approach to interfere with the biological activity of the membrane bound c-fms.

      A monoclonal antibody against the ligand binding domains of the M-CSF receptor (c-fms) was initially utilized by the inventor to inhibit the biological effects of M-CSF in

20   LDLR-deficient mice. Macrophages isolated from LDLR-deficient and control C57BL/6 mice showed positive immunoreactivity to the c-fms monoclonal antibody. Both control and LDLR-deficient mice on a C57BL/6 genetic background of 7 to 10 weeks of age, when injected intraperitonealy with 0.5 mg/day of the c-fms antibody every alternate day, exhibited increased levels of serum cholesterol and decreased numbers of monocytes in

25   circulation as compared to mice injected with either saline or an isotyped antibody control. There were features of osteopterosis noted as well, including mild growth retardation in mice injected with the anti-c-fms monoclonal antibody. When LDLR-deficient mice were fed a pro-atherogenic diet, mean aortic atherosclerotic lesion area was significantly reduced by injecting c-fms antibody compared to their uninjected

30   littermates on the same diet. Analysis of variance between antibody injected vs. uninjected LDLR-deficient mice (n=6) revealed that this difference was significant (p<0.01). The atheromatous lesions in the c-fms antibody injected LDLR-deficient mice

showed highly reduced numbers of monocyte-macrophages as examined by immunohistochemistry. Although the monoclonal antibody based method exhibited effectiveness in reducing atherosclerosis, it was labor-intensive and expensive. It moreover required frequent intravenous injections of c-fms monoclonal antibody, which
5   could limit the practical and widespread applicability of this approach in humans. Alternative means of delivering effective doses of therapeutic c-fms inhibitors were therefore desirable.

One aspect of the present invention evaluates the feasibility and efficacy of somatic cell gene transfer utilizing viral vectors containing c-fms gene sequences that
10   express antisense RNA. Appropriate viral vectors that can express antisense c-fms RNA include expression vectors based on recombinant adenovirus, adeno-associated virus, retroviruses or lentivurses. An ideal vector for c-fms antisense gene transfer against atherosclerosis and angioplasty/stent-induced restenosis in mammals has the following attributes: (1) high efficiency of in vivo gene transfer; (2) recombinant gene expression in
15   dividing as well as nondividing cells (the baseline mitotic rate in the coronary artery wall is <1% even in advanced lesions); (3) rapid and long-lived recombinant gene expression; (4) minimal vascular toxicity from inflammatory or immune responses; (5) absence of baseline immunity to the vector in the majority of the population; and (6) lack of pathogenicity of viral vectors. This is not to say that a vector must have all of these
20   attributes; indeed, many useful vectors will not.

In a preferred embodiment of the invention, one employs adenovirus serotype 5 (Ad5)-based vectors (Quantum Biotechnology, Inc., Montreal, Quebec, Canada) to deliver and express c-fms gene sequences expressing antisense RNA in cultured macrophages and vascular smooth muscle cells and in atherosclerosis-prone mice and
25   swine. The recombinant Ad5 vectors have several advantages over other vectors such as liposomes and retroviruses. Unlike retroviral vectors, proliferation of the target cell is not required for infection by adenovirus vectors and thus, Ad5 vectors can infect cells in vivo in their quiescent state. Ad5 vectors are capable of infecting a number of different tissues although the transduction efficiency can vary according to the cell type. Ad5
30   vectors as a means of in vivo gene delivery have certain drawbacks: (1) gene expression from cells transduced with the Ad5 vector is often transient due to the elimination of the Ad5-transduced cells by the host immune system; (2) Ad5 vectors may

generate some toxicity to human recipients as observed in human clinical trials in cystic fibrosis patients; and (3) initial administration of Ad5 vectors produces blocking antibodies to the vectors, thereby decreasing the effectiveness of repeated administrations. Ad5 vectors are preferred nonetheless, and may be used to effectively

5   practice the methods of the invention described herein.

Using reverse transcripatse polymerase chain reaction (RT-PCR), a portion of *c-fms* mRNA corresponding to 976 nucleotides from the 5'-terminus is isolated and cloned upstream to the human cytomegalovirus (CMV) major immediate early promoter-enhancer in a direction to generate antisense *c-fms* RNA. The use of recombinant Ad5

10   vectors demonstrates the usefulness of gene transfer for the delivery of therapeutic antisense RNA in murine and swine models, and shows that adenovirus-mediated gene therapy can be particularly well suited as an adjunct to coronary angioplasty, since even temporary inhibition of smooth muscle cell proliferation could suffice to limit the formation of restenotic lesions.

15   A second embodiment of the present invention provides a method of gene therapy to produce high levels of soluble forms of membrane bound *c-fms* protein that competes for non-bound M-CSF but lacks all of its signal transduction domain. The three N-terminal immunoglobulin-like domains of the *c-fms* receptor protein constitute the high-affinity M-CSF binding region (the fourth and fifth immunoglobulin-like domains

20   perform functions other than ligand binding). Z. Wang *et al.*, "Identification of the ligand-binding regions in the macrophage colony-stimulating factor receptor extracellular domain," *Mol Cell Biol*, 13:5348-5359 (1993). The method involves cloning of a portion of *c-fms* mRNA corresponding to the three N-terminal immunoglobulin-like domains using a combination of an upstream and a downstream primer. A 976 -nucleotides long

25   amplified cDNA is cloned upstream to the CMV promoter/enhancer in an adenoviral expression plasmid. Using the calcium phosphate transfection method, 293T cell cultures is transfected with 5 mg of soluble *c-fms*/rAd5 plasmid DNA followed by a 48-hour period to allow for expression and secretion of *c-fms* into the cell media. The medium from cells transfected with the soluble *c-fms*/rAd plasmid contains a secreted *c-*

30   *fms* protein concentration up to 33 ng/ml. To increase this relatively low soluble *c-fms* expression, newer versions of rAd vectors that employ different promoters including the human U1 promoter, native human *c-fms* gene promoter or tetracycline-inducible

promoter can be employed. *In vitro* assays shows that the soluble *c-fms* binds M-CSF with high-affinity and inhibits the growth and survival of mouse bone marrow derived macrophages.

5   As with many other type of diseases, therapeutic strategies to treat atherosclerotic disease entail treatment for an extended period of time ranging from months to years. Prolonged and efficient transgene transcription from heterologous promoters is a major consideration for gene therapies. The CMV promoter to drive expression of soluble *c-fms* in the present invention has been popularly used to express a variety of genes; it is, however, often subject to epigenetic silencing as are most promoters and transgenes. In

10   an attempt to circumvent this problem, a variety of promoter expression strategies can be used to optimize the *in vivo* production of the soluble *c-fms* in the present invention. Efficient gene expression in viral vectors depends upon a variety of factors. These include promoter strength, message stability and translational efficiency. Each of these factors must be explored independently to achieve optimal expression of soluble *c-fms*

15   gene. Applications of other promoter/enhancer variants to increase and optimize the expression of soluble *c-fms in vitro* as well as *in vivo* are included within the scope of this invention. These include promoters or enhancers that are stronger than CMV or that exhibit inducibilty. Tetracycline-inducible promoters, for example, or promoters that are tissue-specific, such as vascular-endothelial or smooth-muscle specific

20   promoters/enhancers, may be used to produce sufficient amounts of soluble *c-fms* or its derivative. The presence of soluble *c-fms* or its derivative reduces the amount of M-CSF ligand available for binding and thereby inhibits the M-CSF/*c-fms* cell signaling pathway. Levels and persistence of soluble *c-fms* expression can be compared with those obtained from the CMV promoter.

25   A third embodiment of the invention administers a ribozyme-viral (adeno, adeno-associated or lentiviral) vector against *c-fms* mRNA in a mammal. The method involves development of a hammerhead ribozyme expression cassette in a viral backbone that targets the GUC sequence in codon 18 of *c-fms* mRNA. Ribozymes are sequence-specific endoribonucleases that catalytically cleave specific RNA sequences, resulting in

30   irreversible inactivation of the target mRNA, thereby inhibiting the gene expression. T. Cech. "Biological catalysis by RNA," *Ann Rev Biochem*, 55:599-629 (1986); J.J. Rossi, "Therapeutic ribozymes: principles and applications," *Bio Drugs*, 9:1-10 (1998).

Ribozymes display catalytic activity that lowers the amount required for inhibiting gene expression, and in this way offer an advantage over antisense ODN. Ribozymes can be delivered exogenously or can be expressed endogenously with the use of appropriate promoters in a viral vector. In a preferred embodiment of the invention, one uses a

5   hammerhead ribozyme directed to human *c-fms* mRNA; this ribozyme restricts the growth of peripheral blood derived macrophages *in vitro*. Cationic liposome-mediated transfection of macrophages with ribozymes against *c-fms* causes reduced cell growth and survival as determined by MTT assay. Fluorescent modified ribozymes are used to determine the efficiency of cellular uptake and optimal delivery conditions in cultured

10   human macrophages. The results of the *in vitro* studies provide the basis for the *in vivo* usage of ribozymes to inhibit the growth and survival of macrophages in atherosclerotic lesions. Desired quantity or the length of expression of the ribozyme-viral vector can be readily determined without undue experimentation, as can the most effective and/or convenient route of administration.

15   In a fourth embodiment of the invention, there is provided a non-viral method to inhibit the expression of *c-fms* receptor. This method involves synthesis of pentadecamer (15-mer) ODN corresponding to the sense and antisense sequense of the human *c-fms* mRNA. Pentadecamer ODN are known to bind very strongly to single-stranded regions of the target mRNA (D Jaskuski *et al.*, "Inhibition of cellular proliferation by antisense

20   oligonucleotide to PCNA cyclin," *Science*, 240:1544-1548, (1988)) and thereby to strongly inhibit translation of the mRNA. In a preferred embodiment, four ODN are synthesized on an automated solid-phases synthesizer (Applied Biosystem, Inc., Rockville, MD) and purified using standard protocols. Two of the ODN are complementary to first 15 nucleotides of either the sense (SEQ ID NO. 1) or antisense

25   (SEQ ID NO. 2) strand of human *c-fms* mRNA starting with the initiation codon (AUG). The other two ODN are 50% or less base-pair mismatch (SEQ ID NO. 3, SEQ ID NO. 4) with the antisense mRNA. Inhibition of cell proliferation of peripheral blood derived macrophages by antisense *c-fms* ODN is dose-dependent over the range of 2 to 15 micromolar (μM) over a 24 hour period. A single 7 μM dose of antisense *c-fms* ODN

30   inhibited cell proliferation by 57 to 75% whereas the same concentration of sense ODN inhibited proliferation by only 19%. Control ODN did not show any detectable effects on macrophage cell proliferation. The method shows that growth of cultured macrophages

is inhibited by the interaction of antisense *c-fms* ODN with the endogenous *c-fms* mRNA and provides evidence in favor of the using antsense *c-fms* ODN to inhibit the expression of *c-fms in vivo*, thereby blocking the M-CSF/*c-fms* mediated cell signaling in a mammal.

The vascular delivery of viral vectors expressing antisense *c-fms* RNA, soluble *c-fms* protein or ribozyme and non-viral antisense ODN in the present invention can be accomplished using a wide range of local delivery devices and methods. K. L. March, "Methods of local gene delivery to vascular tissues," *Semin Intervent Cardiol*, 1:215-223 (1996). The rationale behind local delivery is that site-specific delivery of vectors may result in maximal therapeutic efficacy with minimal systemic side effects. These local delivery devices typically entail an endovascular or "inside-out" approach whereby therapeutic agents are delivered to the target site via intravascular catheters or devices. Although gene transfer is demonstrated for each device, most studies of catheter-based gene transfer reveal low efficiency, rapid redistribution of the infused material, and escape of the infusate into the systemic circulation. Recently, several devices with modified needles capable of direct injection into interstitial tissue of either myocardium or vasculature are described. One such approach to local drug delivery is via the nipple balloon catheter, or INFILTRATOR® (InterVentional Technologies, Inc., San Diego, CA). Use of the INFILTRATOR® for intramural delivery of small volumes of high-titer rAd5 is preferred. The INFILTRATOR® catheter offers improved local gene delivery by placing vector particles directly deep within the vascular wall. The INFILTRATOR® catheter is designed to provide direct intramural delivery of agents by mechanical access into the media and inner adventitia, which is achieved using sharp-edged injection orifices mounted on the balloon surface. P. Barath *et al.* "Nipple balloon catheter," *Semin Intervent Cardiol*, 1:43 (1996). This catheter has been used clinically, G. S. Pavlides *et al.*, "Intramural drug delivery by direct injection within the arterial wall: first clinical experience with a novel intracoronary delivery-infiltrator system," *Cathet Cardiovasc Diagn*, 41:287-292 (1997), and has been demonstrated to yield enhanced local transduction efficiency by adenoviral vectors compared with that achievable by endoluminal delivery. T. Asahara *et al.*, "Local delivery of vascular endothelial growth factor accelerates reendothelialization and attenuates intimal hyperplasia in balloon-injured rat carotid artery," *Circulation*, 91:2793-2801 (1995).

In an alternate embodiment, one can utilize perivascular or "outside-in" drug
delivery in the vessel wall by modifying the procedure of periadventitial carotid injury in
the mouse. Oguchi S, et al. "Increased intimal thickening after arterial injury in
hypercholesterolemic apolipoprotein E-deficient mice: finding a novel method,"
5   *Circulation* Supplement I-548,3066, (1997); Dimayuga P, et al. "Reconstituted HDL
containing human apolipoprotein A-1 reduces VCAM-1 expression and neointima
formation following periadventitial cuff-induced carotid injury in apo E null mice'" *Biochem
Biophys Res Commun*, 264:465-468, (1999). Apo E-deficient mice (20-weeks of age, 6
per group) are anesthetized, and the carotid artery is exposed by making a small incision
10   in the side of the neck. In one of the inventor's experiments, a section of artery was
loosely sheathed with a cuff made of a Tygon tube (3.0-mm long, 0.50-mm inner
diameter). A biodegradable biocompatible polymeric material, ATRIGEL®, (Atrix
Laboratories, Ft. Collins, CO), a copolymer of polylactic and polyglycolic acid, was used
for the local delivery of viral particles. 18 % (w/w) polymeric gel in PBS with 1×10^{8} pfu of
15   rAd5 (right) or without rAd5 (left carotid) was applied between the cuff and the vessel
using a syringe and blunt cannula. The gel compound used in the study exists as a free-
flowing liquid below body temperature. When placed in an aqueous environment at or
above body temperature, the viscosity increases and the gel solidifies into a viscous
mass. Once applied to the artery *in vivo*, the polymer gels immediately on contact and
20   the gel is then gradually resorbed in about 14 to 21 days, thereby providing potential use
as a drug depot. The gel releases viral particles that cause a reversal in the effects of
periadventitial injury on neointimal response to carotid injury in apo E-deficient mice.

The introduction of surgical and percutaneous arterial revascularization to treat
atherosclerosis has profoundly altered the clinical management of disease, but has also
25   brought unanticipated problems and unanswered questions. In particular, surgical and
especially percutaneous revascularization may elicit an exaggerated healing response,
which in many respects is similar to the development of de novo atherosclerotic lesions.
This "response to injury" is more proliferative in nature than *de novo* lesion formation, but
may nevertheless lead to restenosis, or even late or abrupt vessel closure, and ultimately
30   result in a failed revascularization attempt. For this and other additional reasons, long-
term clinical studies have documented improved outcomes only in selected patient
subgroups; for those with stable angina pectoris, coronary intervention remains merely

palliative, and does not alter the progression or outcome of the underlying causative disease process.

With balloon coronary angioplasty, restenosis rates of 30%-40% or more have been documented, and certain lesion sites and patient subgroups have been found to be particularly susceptible to restenosis. Intensive research efforts into the cause of restenosis have yielded considerable insight, but as yet no unequivocal treatment to eliminate the problem. Technical innovations in revascularization equipment and techniques have shown some success, but even this has been of limited effectiveness. In particular, the development of the intracoronary stent markedly reduced the incidence of restenosis. With proper stent placement techniques, restenosis rates have been reduced to roughly 10%, and so consequently intracoronary stent placement has largely supplanted balloon angioplasty alone as the interventional coronary treatment of choice. Still, given the rapid proliferation and acceptance of intracoronary stenting, even a 10% restenosis rate results in a very large number of patients in whom the revascularization attempt has been unsuccessful, and for whom other treatment strategies have not been convincingly effective. The same patient frequently needs multiple separate interventions, and ultimately these may not be successful.

Since the arterial response to injury is predominantly mitogenic and neoproliferative in nature, intracoronary irradiation (or intracoronary brachytherapy) has been developed and deployed to attempt to reduce further the numbers of patients who restenose following coronary intervention. Intracoronary brachytherapy has also met with limited success, however, and unfortunately has brought with it two new manifestations of the disease as a side effect: geometric miss and late in-stent thrombosis. It appears likely that these two effects will significantly limit the efficacy of intracoronary brachytherapy as a definitive treatment for restenosis. Thus, a need remains for an effective way to limit or eliminate restenosis following coronary stent placement. Alternatively, if intracoronary brachytherapy is to achieve unequivocal effectiveness in eliminating restenosis following stent placement, a solution to late in-stent thrombosis and geometric miss must be found.

One attractive approach to treating restenosis following stent implantation is by modifying the stent to directly influence the cytokines involved in the response to injury that implanting the stent causes. M-CSF expression is rapidly increased following

arterial injury, and abnormally elevated expression levels persist for several weeks thereafter. However, in mice with a targeted deletion of the gene encoding M-CSF, this increased expression of M-CSF cannot occur, and consequently neointimal proliferation following arterial injury is sharply reduced or even abolished entirely. Thus, inhibition of

5   M-CSF signaling provides an attractive gene therapeutic treatment strategy.

In one embodiment of the invention, one coats a stent with rAd5 expressing *c-fms* inhibitors (e.g. antisense RNA, soluble *c-fms* protein, ribozyme) or a compound containing ODN to inhibit and diminish M-CSF/*c-fms* cell signaling. Any stent known in the art may be used for this purpose; this permits one to employ the method of the

10   invention without increasing the time required to implant the stent, and also will not require significant additional equipment, expertise, hospitalization, or expense. This procedure diminishes the need for repeat hospitalizations and additional interventional procedures. It also ameliorates geometric miss and late in-stent thrombosis following intracoronary brachytherapy.

15

EXAMPLES

The following examples are typical of the procedures that may be used to inhibit M-CSF by disrupting the M-CSF/*c-fms* cell-signaling pathway. Modifications of these examples will be apparent to those skilled in or familiar with such procedures.

20   The use of carefully defined animal models is essential to investigate pathophysiologic mechanisms and to assess therapeutic strategies aimed at modifying the arterial luminal narrowing that often occurs following atherosclerosis or vascular injury (restenosis) in humans. The inventor used both large and small animals to establish the effects of *c-fms* inhibitors on the development and progression of

25   atherosclerostic and restenotic lesions. Atherosclerosis prone apo E and LDLR-deficient mice are extremely useful in this regard as they exhibit very similar atherosclerotic lesions as known in humans. One can extend studies using a mouse model of atheroscelrosis and arterial injury to a large animal model to examine the effects of local gene delivery on the coronary arterial luminal narrowing following balloon overstretch

30   and stent overinflation injury in swine. Swine model for coronary injury offers advantages over other animal models because it resembles to human disease. These advantages include: (1) identical cardiac and coronary size and anatomy; hence, coronary injury can

be induced with clinical angioplasty balloon catheters and stents; (2) similar platelet-coagulation systems; (3) similar mechanics of vascular injury as encountered in clinical angioplasty and stenting, e.g., medial dilatation and/or dissection; (4) balanced contribution of the thrombotic and the proliferative processes to neointimal formation; and

5   (5) similarities in topographic and histologic characteristics of intimal lesions (e.g., non-concentric lesion morphology) Additional similarities of the swine coronary balloon overstretch injury preparation to clinical angioplasty in humans include similar mechanisms of luminal narrowing following the procedure, including elastic recoil (acute remodeling), vasoconstriction, inadequate compensatory enlargement (chronic

10   constrictive remodeling), SMC proliferation and extracellular matrix synthesis. H. Luo *et al.*, "Coronary artery restenosis after balloon angioplasty in humans is associated with circumferential coronary constriction," *Arterioscler Thromb Vasc Biol*, 16:1393-1398 (1996); H. R. Andersen, "Remodeling rather than neointimal formation explains luminal narrowing after deep vessel wall injury," *Circulation*, 93:1716-1724 (1996).

15   **EXAMPLE 1**

Cloning of *c-fms* gene sequences expressing antisense RNA or soluble protein

RT-PCR was performed to amplify *c-fms* gene DNA sequences corresponding to the ligand binding domain of the receptor using specific sets of primers on the DNA template produced from mouse or human macrophage mRNA. Human macrophages

20   were grown from peripheral blood monocytes following standard protocols. Mouse macrophages were derived from bone marrow cells. Total cellular RNA was isolated by lysis of mouse or human macrophages in guanidinium isothiocyanate, phenol-chloroform extraction and ethanol precipitation. P Chomzynski, N Sacchi. "Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal

25   Biochem. 162:156-159 (1987). To amplify the mouse and human *c-fms* gene sequence corresponding to the ligand binding domain (Fig. 1) two primers (cfms-1, SEQ ID NO. 5, nucleotides 75 to 93; and cfms-2, SEQ ID NO. 6, nucleotides 994 to 1008) were synthesized corresponding to the high affinity M-CSF binding region. Z. Wang et al., "Identification of the ligand-binding regions in the macrophage colony-stimulating factor

30   receptor extracellular domain," Mol Cell Biol, 13:5348-5359 (1993). RT-PCR was performed to amplify a 976 nucleotides long cDNA sequence using total RNA prepared from human or mouse macrophages as substrate. The amplified products were

subcloned in pCRII vector (Invitrogen, San Diego, CA). Diagnostic restriction digestion and DNA sequencing were performed to establish the identity of the mouse or human *c-fms* gene sequences.

EXAMPLE 2

5   **Construction and purification of recombinant adenoviral viral vectors expressing either antisense RNA or soluble protein**

The choice of the Ad5 gene delivery system for *c-fms* gene was based on its ease of use, and many attractive features that allow the efficient transfer of genetic material as compared to other viral and non-viral (e.g., "naked" DNA or lipid coated DNA) gene

10   delivery systems. Ad5 produces stable virions with high titers that are capable of transducing replicative and non-replicative cells. Ad5 has a wide host and tissue range and exhibits low pathogenicity in humans.

A 976 base pair EcoRI fragment of mouse *c-fms* cDNA was excised from pCRII plasmid and gel purified. For recombinant adenoviral transfer vector construction, BglII

15   linkers were attached to the 976 base pair fragment comprising the protein coding region of *c-fms* gene. This fragment was then ligated to a BglII linearized transfer vector pQBIAdCMV5-GFP (Quantum Biotechnology, Inc., Montreal, Quebec, Canada) between the strong enhance/promoter of the CMV immediate early genes and the SV40 polyadenylation signal (Fig. 2). The presence of 976-base pair fragment in the transfer

20   vector clones was confirmed by performing PCR using mcfms-1 and mcfms-2 primers. The orientation of the *c-fms* fragment with regard to the CMV promoter enhancer was determined by double restriction digestion of the recombinant adenoviral transfer vectors using BglII and ApaI restriction enzymes. ApaI was a convenient asymmetrical site in the *c-fms* cDNA insert. DNA sequencing was carried out on the clones of the transfer vector

25   that contained mouse *c-fms* fragment. Recombinant transfer vector containing both right and wrong orientation of *c-fms* fragment were individually isolated and purified.

To produce the recombinant viral particle, recombinant adenoviral transfer plasmid was linearized with EcoRI. The linearized DNA was extracted once with buffer-saturated phenol and choloroform/isoamyl alcohol (24:1), precipitated with 1/10 volume

30   of 3M NaAcetate pH 5.2 and 2.5 volumes of ethanol and resuspended in Tris-EDTA buffer. The concentration of the linearized plasmid was estimated on an agarose gel. 5 µg of linearized transfer plasmids containing the *c-fms* gene were mixed with 5 µg of

adenoviral DNA and transfected onto sub-confluent 293 A cells in a 60 mm petridish using the standard calcium phosphate technique. 293A cells line is a permanent line of primary human embryonic kidney cells transformed by sheared Ad5 DNA. This cell lines contains the E1A and E1B, Ad5 genes that complement these genes deleted in the

5   recombinant adenovirus. After overnight incubation, the co-precipitated DNA-calcium phosphate was removed and cells were washed with phosphate-buffered saline (PBS). The cells were split into 6 well plate and left to attach for 6 hours followed by overlayering with agarose following the protocol described in the Adeno-Quest kit supplied by Quantum Biotechnology.

10   Recombinant Ad5 plaques were screened for the expression of the *c-fms* gene sequences and viral stocks of the first amplification were prepared. Each clone was tested for the expression of recombinant protein and/or virus production. A final round of PCR was performed to fully characterize the rAd5 that express either antisense *c-fms* RNA or soluble *c-fms* protein. Large scale production of high titer viral recombinant

15   particles were carried out in 293 A cells. This involved a sequential increase in cell culture size and adenovirus infection cycles. Purification of recombinant Ad5 expressing either antisense *c-fms* (Ad5CMV*c-fms*AS) or soluble protein (Ad5CMV*c-fms*SA) was accomplished by double cesium chloride gradient. Titration of the recombinant viral particle were carried out using a combination of plaque assay method and real time PCR

20   (Applied Biosystems, CA)

EXAMPLE 3

Adenovirus mediated *c-fms* gene transduction in cultured human and mouse macrophages

Macrophages, derived from either from human peripheral blood monocytes or

25   from mouse bone marrow, were transduced with adenoviral vectors expressing antisense *c-fms* RNA (Ad5CMV*c-fms*AS) or soluble *c-fms* protein (Ad5CMV*c-fms*SA) linked to green fluorescence protein (GFP) to allow sorting of positive transfectants. Transfection efficiency of the recombinant virus at different MOI was determined by direct visualization and flow cytometric analysis of GFP fluorescent activity. The

30   repression of *c-fms* in Ad5CMV*c-fms*AS transduced macrophages was assessed 2 to 3 days post transduction by flow cytometry using human or murine *c-fms* specific antibodies. Antisense *c-fms* RNA effectively inhibited the expression of *c-fms* protein as

shown by immunoblot analysis in a dose and time dependent manner. Cultured
macrophages showed reduced growth in response to both antisense c-fms RNA
expression and with the expression of soluble protein as early as 1 day post
transduction. As shown in Fig. 3, by 2 days (48 hours) post transduction with Ad5CMVc-
5   fmsAS there were abundant detachment of macrophages from tissue culture dish. These
cell showed rounding off with continued expression of GFP. MTT cell survival assay
was used to measure changes in cell survival at various time point post transduction.
Results of this experiment show that macrophages were successfully transduced with
adenoviral vectors expressing antisense c-fms RNA (Fig. 3) or soluble c-fms.
10   Expression of these molecules by macrophages was associated with poor growth and
reduced survival (Fig. 4 A and B). Collectively, these results suggest a potent inhibitory
effect of the adenoviral expressed antisense c-fms RNA and soluble c-fms suggesting
that both strategy could be exploited to inhibit the survival and activation of mononuclear
phagocytic cells and atherogenesis.

15   **EXAMPLE 4**

Studies with cultured mouse, pig and human vascular smooth muscle cells

In vitro transducibility of the recombinant viral particles expressing antisense c-fms
transgene was assessed by infecting smooth muscle cells derived from mouse, pig and
human vessel wall (Fig. 5 to 8). Harvesting of vascular SMC from mouse, pig and
20   human were done using standard procedures. Cell viability was assessed by Trypan blue
exclusion. Morphologically typical SMC were observed under the light microscope, and
the immunocytochemical staining of a specific SMC marker α-actin was adapted for the
identification of mouse vascular SMC (Fig. 5). Mouse, pig and human vascular SMC
when transduced with the increasing dose Ad5CMVc-fmsAS showed increased levels of
25   antisense c-fms RNA as determined by RT-PCR (Fig. 6) and Northern blotting assays
(Fig. 7). Transduced mouse and human vascular SMC showed high levels of GFP
expression while untransduced cells lacked detectable fluorescence. As with
macrophages, anisense c-fms RNA inhibited the growth and survival of vascular SMC
(Fig. 5 and 8) in a dose and time dependent manner. By 2 days (48 hours) post
30   transduction with Ad5CMVc-fmsAS there were abundant detachment of vascular SMC
from tissue culture dish. These cell showed rounding off with continued expression of
GFP. Immunostaining for the TUNEL revealed that adenoviral vector dependent

expression of antisense *c-fms* was triggering an accelerated apoptotic process in these
cells. Results of this experiment clearly show that vascular SMC can be successfully
transduced with adenoviral vectors expressing antisense *c-fms* RNA. Expression of
antisense *c-fms* RNA by vascular SMC was associated accelerated programmed cell

5   death in these cells. These findings favor a role for M-CSF in the growth and proliferation
of vascular SMC suggest that adenoviral expressed antisense *c-fms* RNA based strategy
could be utilized to inhibit the accelerated growth of SMC observed in atherosclerotic and
restenotic lesions in humans. The capacity to transduce human coronary SMC opens up
the possibility of directly targeting the cells of the arterial wall in inhibiting and/or

10   reversing the development of atherosclerotic or restenotic lesions. The results from this
experiment indicate that the encapsidated rAd vectors expressing antisense *c-fms* RNA
are capable of transducing swine VSMC and provide support to study the effects of
antisense *c-fms* gene therapy on injury-induced luminal narrowing in swine.

EXAMPLE 5

15   **Inhibition of atherosclerosis by antisense *c-fms* RNA and soluble *c-fms* protein**
expressed *in vivo* from recombinant Ad5 vectors in the apo E-deficient mouse
model

The effectiveness of antisense *c-fms* gene transfer on atherosclerosis was
assessed by intravenous IV injection of rAd5 expressing antisense *c-fms* RNA or soluble

20   *c-fms* protein in apo E-deficient mice (5 weeks of age) at a 1×10^8 pfu per animal dose.
One group (10 animals per group) of apo E-deficient mice were given the rAd5 vector
with the antisense *c-fms* transgene linked to eGFP reporter and a second group of apo
E-deficient mice were administered the rAd5 vector encoding soluble *c-fms* protein linked
to eGFP reporter. Control groups of apo E-deficient mice included (i) untreated mice, (ii)

25   mice receiving a control rAd5 vector with the eGFP gene. Five weeks after the
administration of the recombinant virus, one set of animals from all groups were
sacrificed and aortas were harvested. In addition, relevant tissue (liver spleen, lung,
kidney, testes, and vessel,) were obtained from the dead animals to analyze for the
expression of antisense *c-fms* RNA (Fig. 9), soluble *c-fms* mRNA, host chromosome

30   integration of the transgenes and host immune response to the rAd5 vectors. For this
first set of animals, blood samples were collected at 0, 4, 14, and 35 days after the
injections. Ten weeks after administration of the recombinant virus, a second set of mice

of all groups were sacrificed and the aforementioned protocol were repeated. For this second set of mice, blood samples will be collected at 0, 4, 14, 35 , 56 and 70 days after the injections. Mice were on a cholesterol-rich diet throughout the experiment.

5

Following anesthesia with enflurane, mice were euthanized and their hearts and aortas were perfusion-fixed with 4% paraformaldehyde, 5% sucrose, 20 mM EDTA at physiologic pH for 10 minutes. The heart and the proximal aorta were then excised and embedded in OCT compound (Tissue-Tek), frozen on dry ice, and then stored at -70°C until sectioning. Serial 10-μm thick sections, every fifth section from the middle of the ventricle until the appearance of aortic valve and every second section from the

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appearance to the disappearance of the aortic valve leaflets, were collected on poly-D-lysine-coated slides and stained with Oil Red O and hematoxylin and counter stained with fast green. Quantitation of the Oil Red O stained area in the aortic sinus plaques will be performed by computer-assisted morphometry using IMAGE PRO software and expressed as average of lesion areas from six sections per mouse. The remainder of

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the thoracic and abdominal aorta (up to bifurcation of common iliac arteries) were removed, stored overnight in formal-sucrose fixative, cut open longitudinally, and stained with Sudan IV to visualize the extent of atherosclerosis of the entire aorta. Quantification of the percentage of aortic surface covered by atheromata were accomplished by computer-assisted planimetry of the Sudan IV-positive areas. In selected sections of the

20

aortic sinus plaques, immunohistochemical staining were performed using monoclonal rat anti-mouse Mac-1 antibody (Boehringer Mannheim Biochemicals) as a marker for mouse monocyte/macrophages and the area of plaque occupied by Mac-1 immunoreactivity were measured using image analysis software described above.

25

Serum samples were analyzed for total serum cholesterol by enzymatic technique and after precipitation of apo B-containing lipoprotein with phosphotungstic acid, serum HDL cholesterol concentrations were determined. In addition, Northern and Southern blot analyses were performed to determine the tissue expression of the antisense c-fms RNA and the soluble c-fms mRNA and the possible integration of the recombinant viral vector, respectively.

30

Results of this experiment indicate that rAd5 mediated transfer of mouse c-fms gene induced expression of antisense c-fms RNA and soluble c-fms in sufficient quantities, for sufficient duration and with sufficient functionality that prevented the

progression of atherosclerosis or favourably changed the composition of atherosclerotic plaques (lipid and macrophage depletion) in the apo E-deficient mouse model. A comparison of the outcome of apo E-deficient mice treated with the antisense transgene with that of mice injected with the sequences encoding soluble *c-fms* revealed that

5   antisense RNA was more potent in providing protection against atherosclerosis (Fig. 10 and 11).

EXAMPLE 6

Inhibition of atherosclerosis by antisense *c-fms* RNA expressed *in vivo* from Ad5 vectors in the LDLR-deficient mouse model

10   The inventor evaluated the inhibitory effects of antisense *c-fms* RNA *in vivo* in LDLR-deficient mice. Mutant mice fed a high cholesterol diet were given intravenous injections of Ad5CMV*c-fms*AS linked to a reporter eGFP gene (1x10e8 pfu in 100 ul volume) beginning at 20 weeks of age with a booster of same dose at 10 days postinjection of Ad5CMV*c-fms*AS. Mice were sacrificed five weeks after vector injection.

15   Compared to untreated or vector (Ad5CMVpolyA linked to eGFP reporter) only treated mice, Ad5CMV*c-fms*AS -treated mice showed a lack of progression of aortic atherosclerotic lesion despite persistent and marked hypercholesterolemia (Fig 12). Atherosclerotic lesion area in the aortic arch was 30+ 5% at the time of vector injection as measured by Oil Red O staining. Lesioned area in mice receiving Ad5CMVpolyA

20   progressed to 52+4% (n = 4) whereas lesion size in mice receiving Ad5CMV*c-fms*AS was 21+3% (p<0.05 vs. rAdGFP, n = 4). The antiatherogenic effects of Ad5CMV*c-fms*AS were associated with lipid and macrophage depletion from atherosclerotic plaques. There was no evidence of an adverse response with the use of recombinant Ad5CMV*c-fms*AS except mild reduction in the number of monocytes. Taken together, these studies

25   indicate that in LDLR null mice, *c-fms* antisense gene transfer reduces progression of atherosclerosis and favorably alters plaque phenotype despite augmented hypercholesterolemia.

EXAMPLE 7

Safety of systemic administration of rAd5 expressing antisense *c-fms* RNA or

30   soluble *c-fms* protein

          Histologic examination of areas of injection and various tissues and organs (liver, lung, kidneys, etc.) were performed to detect for the presence of inflammation, necrosis

and/or tumor formation. Frozen tissue samples from mice treated with the *c-fms*
sequences encoding either antisense RNA or soluble *c-fms* well as the control groups
were cryostat sectioned at 20 µm thickness and stained with hematoxylin-eosin (H&E).
The H&E-stained tissue were examined for the presence of infiltrates.

5   In order to determine if a humoral immune response may be occurring in animals
injected with the recombinant viruses, serum from animals administered with Ad5CMV*c-fms*AS, Ad5CMV*c-fms*SA and Ad5 vectors were tested for inhibition of cellular
transduction *in vitro*. Using COS or CV-1 cells, serial dilutions of the serum samples were
added to their respective recombinant viral particles (rAd5) prior to transduction of the
10   cells. The neutralizing titers of the serum samples were determined by their ability to
inhibit transduction *in vitro*. In order to determine the possible spread of the rAd5 vector
to other tissue, tissue samples including gonadal tissue were examined using PCR
analysis using specific primers for the antisense *c-fms* gene.

EXAMPLE 8

15   **Effect of rAd5-mediated transfer of antisense *c-fms* transgene on arterial response
to injury in the carotid injury model in apo E- deficient mice**

A model of carotid artery injury in the apo E-deficient mouse that results in marked
neointimal thickening (S. Oguchi *et al.*, "Increased intimal thickening after arterial injury in
hypercholesterolemic apolipoprotein E-deficient mice: finding a novel method,"
20   *Circulation* , I-548, 3066 (1997), was used to examine the effects of rAd5-mediated
transfer of antisense *c-fms* transgene on arterial response to injury. The carotid injury
was induced by placing a cuff consisting of Tygon tubing around the artery in
anesthetized apo E-deficient mice. Three weeks later, substantial neointimal thickening
is observed in the segment of carotid artery encased in the cuff. Using this model, the
25   effect of M-CSF deficiency in *op/op* mice on the neointimal response to injury was
previously studied. A total absence of M-CSF was associated with minimal or no
neointimal thickening compared to control apo E-deficent mice findings suggesting that
M-CSF-mediated cell signalling either directly or indirectly critically contributes to the
neointimal response to injury in mice.

30   In order to test critically the vascular effects of antisense *c-fms* RNA locally in the
arterial wall, the inventor used the perivascular delivery system to transduce the carotid
artery of apo E-deficient mouse with an Ad5CMV*c-fms*AS virus expressing antisense *c-*

fms RNA whose expression was linked to GFP reporter gene. Following the established protocol a dose of Ad5CMV*c-fms*AS viral vector containing 5×10^6 PFU was prepared in 18 % (w/w) polymeric gel (dissolved in PBS). The gel with virus was applied to the right carotid artery between the cuff and the vessel. Untreated left carotid in the same animal

5   served as a control. Mice were sacrificed 14 days post transduction and their carotids were processed to visualize GFP. Result of one such experiment is shown in Fig. 13. There was abundant expression of GFP reporter throughout the vessel wall (right panel) in the adenovirus transduced artery as compared to untreated left artery (left panel) indicating local release of viral particles within the arterial wall.

10   **EXAMPLE 9**

Evaluation of the effects of local rAd5-mediated transfer of antisense c-*fms*
transgene on coronary arterial luminal narrowing response to
balloon-overstretch injury in pig

M-CSF ligand deficiency in mice exhibits potent vascular antiproliferative

15   properties. rAd5-mediated intramural gene transfer has the potential to provide enhanced delivery efficiency, prolonged recombinant gene expression, and minimization of immune and inflammatory responses. Overexpression of antisense c-fms transgene by intramural delivery system may therefore be a promising method for therapeutic modulation of postinterventional coronary restenosis. An additional advantage is the

20   targeting of the adventitia, which plays a critical role in the coronary arterial response to injury by influencing neointimal formation as well as remodeling.

The subject animals are adult domestic swine weighing 25-30 kgs. Animals are fed a normal diet and housed in a vivarium. After an overnight fast, swine are pretreated with oral aspirin (325 mg) the day prior to coronary artery injury. The animals are

25   immobilized by an IM injection of acepromazine (0.5 mg/kg), ketamine (20 mg/kg), and atropine (0.05 mg/kg), and anesthesia induced with IV thiopental (5-8 mg/kg) and maintained by 1-2% isoflorane after endotracheal intubation. The animals are given bretylium tosylate (250 mg IV) and heparin (5000 U IV) prior to coronary instrumentation. Mechanical ventilation, arterial BP and continuous ECG monitoring is performed

30   throughout the procedure.

Following surgical exposure, an 8F sheath is inserted into the left carotid artery, and an 8F AL-0.75 guiding catheter is advanced to the ostia of the coronary arteries

under fluoroscopic guidance. After administration of intracoronary nitroglycerin (200 µg) and angiography to estimate the size of the vessel, balloon injury is performed either on the left anterior descending or the right coronary artery with angioplasty balloons (3.5-4.0 mm in diameter, 20 mm in length) sized so that the ratio of inflated balloon to artery is ~

5   1.3:1. The balloon is inflated to 8-10 atmospheres for 30 seconds three times, with 60 seconds intervals between each inflation. The (ACT-one are then deployed in the other coronary artery in segments averaging 2.7 to 3.0 mm in diameter, using the location of diagonal or septal branches as reference. All stents are hand crimped on a 3.0mm or 3.5 mm balloon to obtain a final stent:artery ratio of ~ 1.3:1. The balloon is inflated twice

10   to 6-8 atm for 20-30 seconds to deploy the stent. Angiography is repeated to confirm injury, evidenced by an obvious "step-up" and a "step-down" of the injured segment on visual inspection of the angiogram performed after stent placement.

Following coronary injury, the INFILTRATOR® catheter (InterVentional Technology Inc.) is used for rAd5-mediated gene transfer to the site of injury. The

15   INFILTRATOR® is a triple-lumen balloon catheter allowing intramural drug delivery via 3 longitudinal strips of 6 or 7 low-profile 0.254-mm injector ports capable of penetrating the internal elastic lamina (IEL). The catheter allows local drug delivery without perforation, dissection, or hemorrhage of the arterial segment. The inflation of the 3.0-mm balloon at 2 atm is followed by manual injection of 0.3 mm of the viral solution over a period of 15

20   seconds. The viral solutions are injected following balloon overstretch injury and prior to stent-implantation injury. The catheters are withdrawn, the carotid artery ligated, and the skin incision closed. As prophylaxis against infection, all animals receive antibiotics at the end of the procedure. The animals are allowed to recover from anesthesia, returned to the vivarium and fed a normal chow diet and aspirin (325 p.o. mg) continued daily until

25   sacrifice, and in addition are given ticlopidine (250mg p.o. daily) for about 1wk.

Follow-up coronary angiography and intravascular ultrasonography (IVUS) are obtained at specific time intervals (as described in the experimental protocol), following which animals are euthanized by an overdose of pentobarbital (50 mg/kg IV) under general anesthesia. The heart is excised after thoracotomy, and the coronary arteries

30   are perfused first with saline to clear the blood, then perfusion-fixed with 2% paraformaldehyde for 15 minutes followed by immersion in 4% paraformaldehyde in phosphate buffer (pH 7.4) for 4 hours, and then stored in 70% ethanol. To preserve the

integrity of the adventitial and perivascular tissues, coronary arteries are carefully removed along with adjacent tissues (the surrounding adipose tissue and myocardium). The arteries are sectioned into 3-mm blocks from three segments (proximal, middle and distal) of the injured portion of each artery, embedded in paraffin, cut into 15 µm-thick

5   sections, and then stained with hemotoxylin and eosin, and elastin stain for histomorphometric analysis. For stented segments, special histologic processing is performed to maintain the vascular architecture with the metallic stent struts intact . Tissue blocks are cut with a diamond wafering blade and embedded in methyl methacrylate. Two radial cross sections containing 12 struts are cut; one from the

10   proximal and one from the distal half of each stent. Sections are ground to a thickness of about 50 µm, optically polished, and stained with toluidine blue (paragon stain).

Quantitative coronary angiography (QCA) is used to assess minimum luminal diameter (MLD) at specific time points (pre-injury, immediately post-injury, and 28 days following injury immediately prior to euthanasia. Late luminal loss is calculated as the

15   difference in the mean luminal diameter (MLD) immediately post-balloon injury and at 28 days following injury. Remodeling index is calculated as late luminal loss divided by post-injury MLD. Percent stenosis of the injured segments is also estimated using the uninjured segments as reference. The following (IVUS) parameters are measured: stent area, intimal area, % area stenosis, luminal loss, and % diameter stenosis.

20   A computerized imaging system (Optimas) is used for histomorphometric measurements of: 1) mean cross-sectional area and thickness of lumen (area circumscribed by the intima/neointima-luminal border); neointima (area between the lumen and the IEL, and when the IEL is missing, the area between the lumen and the remnants of media or the external elastic lamina [EEL]); media (area between the IEL

25   and the EEL); vessel size (area circumscribed by the EEL but excluding the adventitial area); adventitia (area between the periadventitial tissues, adipose tissue and myocardium, and the EEL); and 2) Injury score. To quantify the degree of vascular injury, a score based on the amount and length of tear of the different wall structures is used. The degree of injury is calculated as follows: 0 – intact IEL; 1 – ruptured IEL with

30   exposure to superficial medial layers; 2 – ruptured IEL with exposure to deeper medial layers (medial dissection); 3 – ruptured EEL with exposure to the adventitia. The injury score for each of the three segments is averaged to obtain the mean artery injury score.

The circumference of the IEL and the length of its rupture, when present, are measured, as are minimal and maximal thicknesses of the neointima, media and adventitia. The relationship between the neointimal and adventitial responses, luminal dimensions, and the extent of injury will also be plotted.

5   The Vectastain Elite ABC system (Vector Laboratories) will be used for immunohistochemistry to identify proliferating cell nuclear antigen (PCNA) to assay the degree of S-phase activity and macrophage infiltration. Cell density and cell proliferation index is determined by counting total cell nuclei and PCNA-positive nuclear staining, respectively, in a minimum of 200-300 cells per vessel layer per field. Cellularity is

10   expressed as number of cells per mm^2, and proliferation index as percentage of PCNA-positive cells. Macrophage content is quantified as macrophage-stained area as a percent of total neointimal area.

In vivo, soluble *c-fms* expression is evaluated by immunohistochemistry on frozen arterial sections. Coronary arterial rings of swine infected with rAd5-soluble *c-fms* is

15   harvested on day 7 (immunocytochemistry protocol) or day 28 (histomorphometry protocol). Sections are incubated overnight with a monoclonal mouse anti-human soluble *c-fms* IgG antibody, followed by incubation with polyclonal goat anti-human soluble *c-fms* IgG for 30 minutes in phosphate buffer containing 1% H_2O_2. Biotinylated rabbit anti-mouse antibody is used as a secondary antibody (Vector Laboratories Inc),

20   and antibody binding is visualized by the avidin-biotin complex method (ABC kit, Vector Laboratories, Inc). For cell-type identification, antibodies specific to SMC α-actin (Boehringer), endothelial cell von Willebrand factor (DAKO), and macrophage CD68 antigen (DAKO) are applied to adjacent sections. Primary antibodies are localized with appropriate Biotinylated secondary antibodies and tertiary avidin-biotin-complex staining

25   (Vector Laboratories). Sections are counterstained with hematoxylin or methyl green and examined by light microscopy.

The following is used to assess gene transfer efficiency. Irrelevant rAd5-placental alkaline phosphatase (PLAP) gene transfer (0.3 ml of 10^{11} infectious units/ml), using the CMV and U1 promoter, is performed via the INFILTRATOR® catheter in native uninjured

30   and balloon-injured arteries (in the same animal) at day 0. Animals are euthanized on day 7 (n=3 each), 14 (n=4 each), and 28 (n=3 each), and the coronary arteries are cannulated, perfused with 4% formaldehyde at 100 cm H_2O pressure for 2 hours, and

washed with PBS for 24 hours. To identify transduced cells expressing the transgene, arteries are cut into 5-mm rings and prepared for alkaline phosphatase histochemical analysis using previously described methods. The entire vessel length will be scanned, and the medial and adventitial cells expressing the transgene are identified by positive stain for PLAP.

The following experimental groups are studied: 1) Histomorphometry (day 28): 0.3 ml of 10^9 and 10^{11} infectious units/ml of rAd5-soluble *c-fms* (8 balloon-injured and 8 stent-injured arteries each for each of the two doses, n=16 total arteries); 0.3 ml of 10^9 and 10^{11} infectious units/ml of rAd5-soluble *c-fms*$_{wt}$ (n=16); and 0.3 ml of 10^{11} infectious units/ml of rAd5-control (n=8). The total number of animals studied in this group will be 40. 2) Immunocytochemistry (day 7): 0.3 ml of 10^{11} infectious units/ml of rAd5-soluble *c-fms* (5 balloon-injured and 5 stent-injured arteries, n=5 arteries total); 0.3 ml of 10^{11} infectious units/ml of rAd5-soluble *c-fms* (n=5); and 0.3 ml of 10^{11} infectious units/ml of rAd5-control (n=5). The total number of animals studied in this group is 15. The total number of animals studied = 75; the total amount of rAd5 required for these studies is 12 ml of 10^{12} infectious particles per ml.

Following baseline angiography, each animal undergoes balloon overstretch injury in one coronary artery (right coronary artery [RCA] or LAD) and stent implantation injury in the other artery in a randomized fashion. Thus, the relative impact of remodeling and hyperplasia on vascular response to injury is examined in the same animal but in different arteries. Unlike the balloon overstretch model where there is a wide variation in vascular injury, the stent overinflation model is a more robust model because the injury is more severe but consistent, resulting in a more exuberant proliferative response and greater associated neointimal formation. Following injury, each artery is infiltrated with the active gene or a control infusate. The animals are euthanized at day 28 after an angiogram and IVUS, and tissues prepared for histomorphometric analysis. In some animals, immunohistochemistry for PCNA and macrophage infiltration is performed at day 7, as cell proliferation and inflammation peak around this time in this model.

CLAIMS

What is claimed is:

1.   A method of inhibiting macrophage colony stimulating factor (M-CSF), the method comprising the steps of

providing a recombinant viral vector comprising a nucleic acid expressing antisense *c-fms* RNA; and

administering the recombinant viral vector to a mammal in an amount effective to at least partially inhibit the M-CSF.

2.   The method of claim 1, wherein the step of administering the recombinant viral vector further comprises administering the vector to a mammal with a vascular disease in an amount effective to inhibit the vascular disease.

3.   The method of claim 2, wherein the vascular disease is selected from the group consisting of atherosclerosis, angina, myocardial infarction, stroke, transient ischemic attacks, osteonecrosis, ischemic colitis, peripheral vascular disease, claudication, renal disease, congestive heart failure, and complications of diabetes.

4.   The method of claim 1, wherein the recombinant viral vector is operatively associated with a promoter.

5.   The method of claim 4, wherein the promoter is U1.

6.   The method of claim 4, wherein the promoter is a tissue-specific promoter.

7.   The method of claim 1, the method further comprising administering the viral vector by intramural delivery.

8.   The method of claim 7, the method further comprising administering the viral vector with a nipple balloon catheter.

9.   The method of claim 1, the method further comprising administering the viral vector by periadventitial delivery.

10.   The method of claim 1, the method further comprising administering the viral vector by implanting within the mammal a stent coated with the viral vector.

11.   The method of claim 1, wherein the recombinant viral vector is administered at a dose of from about 1×10^8 to about 5×10^{16} viral vector genomes per mammal.

12.   The method of claim 1, wherein the mammal is a human.

13.   The method of claim 2, wherein the vascular disease is restenosis following a procedure to reduce atherosclerosis.

14.   The method of claim 13, wherein the procedure is selected from the group consisting of angioplasty and balloon angioplasty.

15.   The method of claim 1, wherein the viral vector is selected from the group consisting of an adenovirus, an adeno-associated virus, a retrovirus, and a lentivirus.

16.   A method of inhibiting macrophage colony stimulating factor (M-CSF), the method comprising the steps of

providing a recombinant viral vector comprising a nucleic acid encoding soluble *c-fms* protein; and

administering the recombinant viral vector to a mammal in an amount effective to at least partially inhibit the M-CSF.

17.   The method of claim 16, wherein the step of administering the recombinant viral vector further comprises administering the vector to a mammal with a vascular disease in an amount effective to inhibit the vascular disease.

18.   The method of claim 17, wherein the vascular disease is selected from the group consisting of atherosclerosis, angina, myocardial infarction, stroke, transient ischemic attacks, osteonecrosis, ischemic colitis, peripheral vascular disease, claudication, renal disease, congestive heart failure, and complications of diabetes.

19.   The method of claim 16, wherein the recombinant viral vector is operatively associated with a promoter.

20.   The method of claim 19, wherein the promoter is U1.

21.   The method of claim 19, wherein the promoter is a tissue-specific promoter.

22.   The method of claim 16, the method further comprising administering the viral vector by intramural delivery.

23.   The method of claim 22, the method further comprising administering the viral vector with a nipple balloon catheter.

24.   The method of claim 16, the method further comprising administering the viral vector by periadventitial delivery.

25.   The method of claim 16, the method further comprising administering the viral vector by implanting within the mammal a stent coated with the viral vector.

26.   The method of claim 16, wherein the recombinant viral vector is administered at a dose of from about 1×10^8 to about 5×10^{16} viral vector genomes per mammal.

27.   The method of claim 16, wherein the mammal is a human.

28.   The method of claim 17, wherein the vascular disease is restenosis following a procedure to reduce atherosclerosis.

29.   The method of claim 28, wherein the procedure is selected from the group consisting of angioplasty and balloon angioplasty.
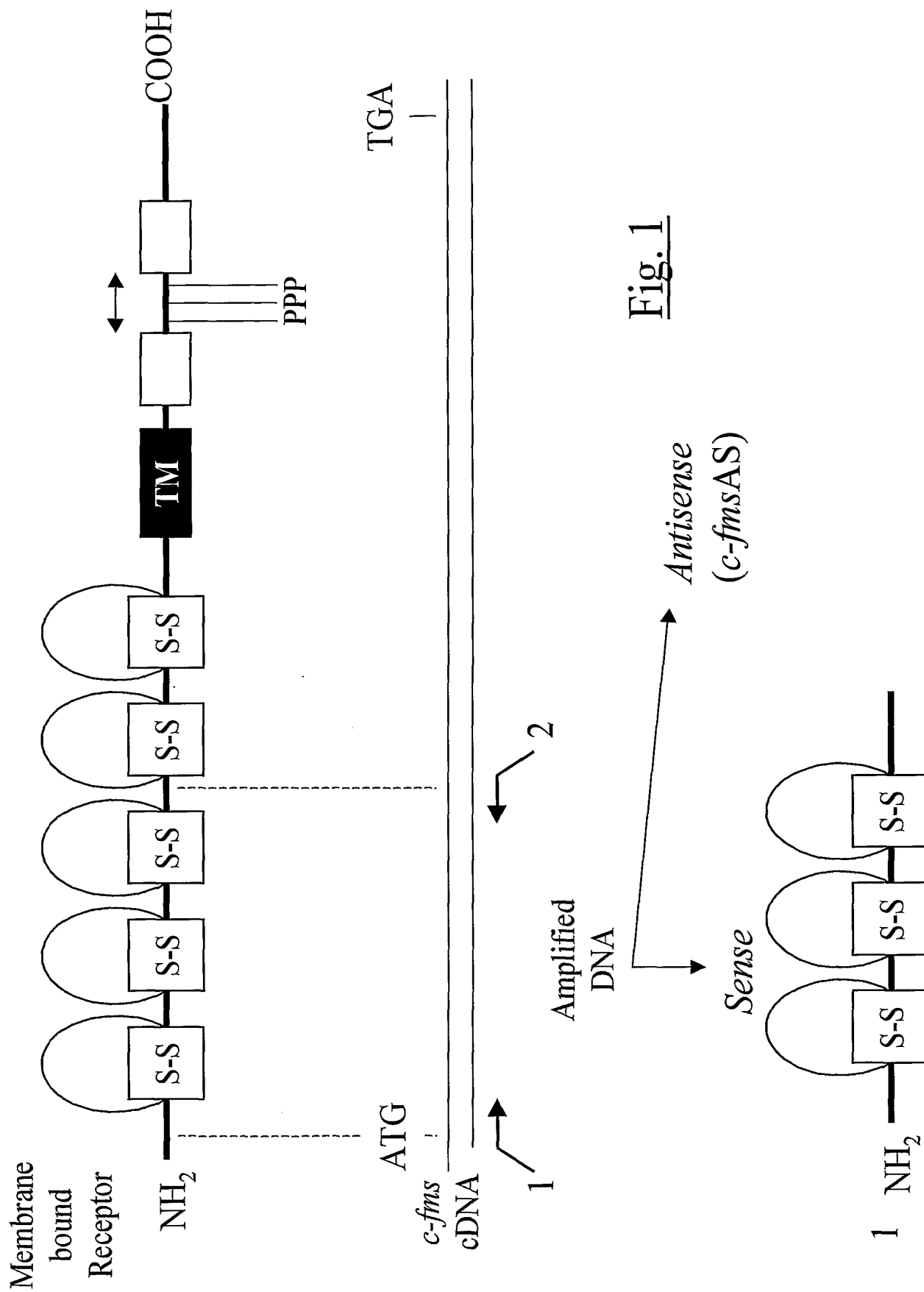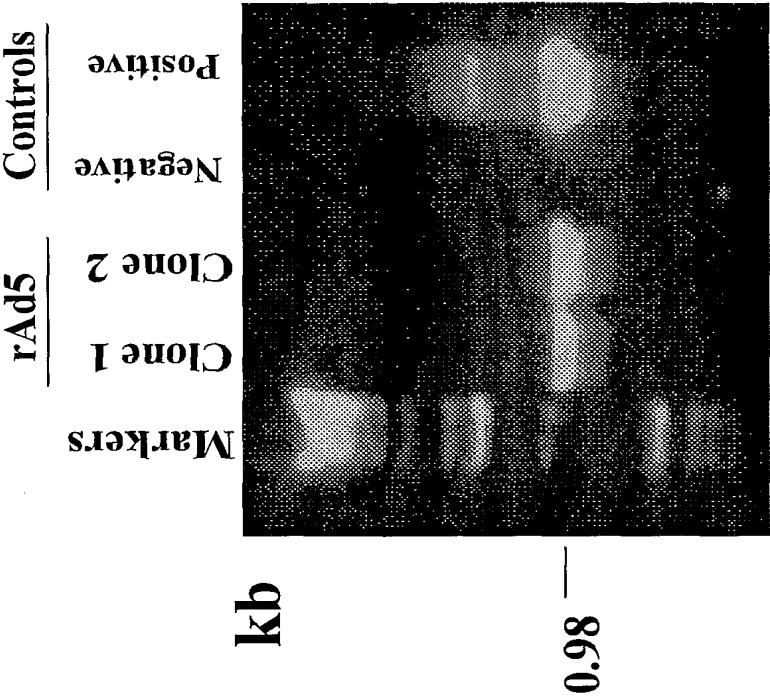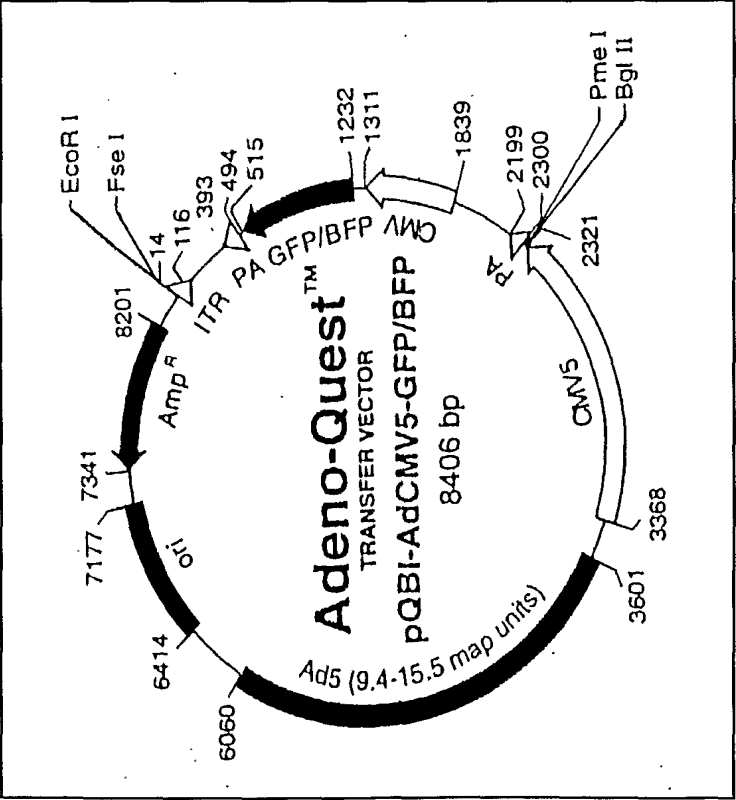
30.   The method of claim 16, wherein the viral vector is selected from the group consisting of an adenovirus, an adeno-associated virus, a retrovirus, and a lentivirus.

31.   A method of inhibiting macrophage colony stimulating factor (M-CSF), the method comprising the steps of

providing a recombinant viral vector comprising a nucleic acid encoding a hammerhead ribozyme that cleaves *c-fms* mRNA; and

administering the recombinant viral vector to a mammal in an amount effective to at least partially inhibit the M-CSF.

32.   The method of claim 31, wherein the step of administering the recombinant viral vector further comprises administering the vector to a mammal with a vascular disease in an amount effective to inhibit the vascular disease.

33.   The method of claim 32, wherein the vascular disease is selected from the group consisting of atherosclerosis, angina, myocardial infarction, stroke, transient ischemic attacks, osteonecrosis, ischemic colitis, peripheral vascular disease, claudication, renal disease, congestive heart failure, and complications of diabetes.

34.   The method of claim 31, wherein the recombinant viral vector is operatively associated with a promoter.

35.   The method of claim 34, wherein the promoter is U1.

36.   The method of claim 34, wherein the promoter is a tissue-specific promoter.

37.   The method of claim 31, the method further comprising administering the viral vector by intramural delivery.

38.   The method of claim 37, the method further comprising administering the viral vector with a nipple balloon catheter.

39.   The method of claim 31, the method further comprising administering the viral vector by periadventitial delivery.

40.   The method of claim 31, the method further comprising administering the viral vector by implanting within the mammal a stent coated with the viral vector.

41.   The method of claim 31, wherein the recombinant viral vector is administered at a dose of from about 1×10^8 to about 5×10^{16} viral vector genomes per mammal.

42.   The method of claim 31, wherein the mammal is a human.

43.   The method of claim 32, wherein the vascular disease is restenosis following a procedure to reduce atherosclerosis.

44.   The method of claim 43, wherein the procedure is selected from the group consisting of angioplasty and balloon angioplasty.

45.   The method of claim 31, wherein the viral vector is selected from the group consisting of an adenovirus, an adeno-associated virus, a retrovirus, and a lentivirus.

46.   A method of inhibiting macrophage colony stimulating factor (M-CSF), the method comprising the steps of

providing antisense c-fms oligodeoxynucleotides (ODN); and

administering the antisense c-fms ODN to a mammal in an amount effective to at least partially inhibit the M-CSF.

47.   The method of claim 46, wherein the step of administering the antisense c-fms ODN further comprises administering the antisense c-fms ODN to a mammal with a vascular disease in an amount effective to inhibit the vascular disease.

48.   The method of claim 47, wherein the vascular disease is selected from the group consisting of atherosclerosis, angina, myocardial infarction, stroke, transient ischemic attacks, osteonecrosis, ischemic colitis, peripheral vascular disease, claudication, renal disease, congestive heart failure, and complications of diabetes.

49.   The method of claim 46, the method further comprising administering the antisense c-fms ODN by intramural delivery.

50.   The method of claim 46, the method further comprising administering the antisense c-fms ODN with a nipple balloon catheter.

51.   The method of claim 46, the method further comprising administering the antisense c-fms ODN by periadventitial delivery.

52.   The method of claim 46, the method further comprising administering the antisense *c-fms* ODN by implanting within the mammal a stent coated with the antisense *c-fms* ODN.

53.   The method of claim 46, wherein the antisense *c-fms* ODN is administered at a dose of from about 7 to about 15 µM per mammal.

54.   The method of claim 46, wherein the mammal is a human.

55.   The method of claim 47, wherein the vascular disease is restenosis following a procedure to reduce atherosclerosis.

56.   The method of claim 55, wherein the procedure is selected from the group consisting of angioplasty and balloon angioplasty.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6A

Fig. 6B

MTT Absorbance

PFU/ml	Ad5CMVpolyA	Ad5CMVc-fmsAS
0	1.296	1.857
10	1.318	2.073
10^2	1.113	1.716
10^3	1.036	1.272
10^4	1.085	0.720
10^5	1.287	0.671

Fig. 7

Fig. 8

Fig. 9

Fig. 10

Fig. 11

PBS

Ad5CMVpolyA

Ad5CMVc-fmsAS

Fig. 12

Fig. 13

Ad5CMVc-fmsAS

Ad5CMVpolyA

Fig. 14

c-fms
mRNA

5'— GCUUGGCAUGGUC   AGGGAAUCCCAGUGA   —3'
      AACCGUACCA   UCCCUUAGGG
                              A C
                              A U
                              G A G
                              C G G
                              A U U
                              G C
                              G C
                              A G
                              G U

1   18   GUC
AUG   TGA

A.

Fig. 15

Fig. 16A

Fig. 16B

Fig. 17

(54) Title: METHODS FOR INHIBITING MACROPHAGE COLONY STIMULATING FACTOR AND C-FMS-DEPENDENT CELL SIGNALING

(57) Abstract: Described herein are methods of inhibiting M-CSF activity, and, in particular, M-CSF/c-fms dependent cell signaling. In a first embodiment of the invention, one administraters to a mammal viral vectors that deliver genes expressing antisense c-fms RNA; in a second embodiment, one induces in vivo production of a high-affinity soluble c-fms protein that competes for non-bound M-CSF; in a third embodiment, one administers a ribozyme-viral vector against c-fms mRNA; and in a fourth embodiment, one administers oligodeoxynucleotides that inhibit expression of c-fms gene product. The methods may be used to treat any disease in which M-CSF activity plays a role, and are particularly effective in treating and preventing atherosclerosis. Embodiments of the present invention are directed primarly, but not exclusively, to a method for treating and preventing cardiovascular disease by inhibiting receptors to M-CSF. Other embodiments of the present invention include any and all biologic and/or pathobiologic phenomena mediated in whole or in part by M-CSF singaling through its receptor. Pathobiologic phenomena inlcude, but are not limited to, disease entities such as osteoprosis, Alzheimer's disease, diabetes mellitus (Type 1 and/or Type 2), infectious disease, cancer, and inherited disorders characterized by defects in one or more components in the M-CSF signaling pathway.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/12251

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) :A61K 48/00; C07H 21/02, 21/04; C12N 15/85, 15/86; C12Q 1/68
US CL : 435/6, 91.1; 514/44; 536/24.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/6, 91.1; 514/44; 536/24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN, MEDLINE, CAPLUS, LIFESCI, EMBASE, USPATFULL, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BORYCKI, ET AL. Repression of the CSF-1 receptor (c-fms proto-oncogene product) by antisense transfection induces G1-growth arrest in L6a1 rat myoblasts. Oncogene. 04 May 1995, Vol. 10, No. 9, pages 1799-1811.	1-56
Y	HIMES ET AL. A highly conserved c-fms gene intronic element controls macrophage-specific and regulated expression. Journal of Leukocyte Biology. November 2001, Vol. 70, No. 5, pages 812-820.	1-56
Y	BORYCKI ET AL. Colony-Stimullating Factor 1 (CSF-1) Is Involved in an Autocrine Growth Control of Rat Myogenic Cells. Experimental Cell Research. May 1995, Vol. 218, No. 1, pages 213-222.	1-56

☒ Further documents are listed in the continuation of Box C.	☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 SEPTEMBER 2002	09 DEC 2002
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer KAREN A. LACOURCIERE
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)★

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	INABA ET AL. ZTranscription Factor PU.1 Mediates Induction of c-fms in Vascular Smooth Muscle Cells: a Mechanism for Phenotypic Change to Phagocytic Cells. Molecular and Cellular Biology. May 1996Vol. 16, No. 5, pages 2264-2273.	1-56
Y	WU ET AL. The role of the c-fms oncogene in the regulation of HL-60 cell differentiation. Oncogene. June 1990, Vol. 5, No. 6, pages 873-877.	1-56
A	INABA ET AL. Involvement of Transcription Factor PU.1 in Phenotypic Transformation of Vascular Smooth Muscle Cells to Macrophage-like Cells. Domyaku Koka. January 1996, Vol. 24, No. 4/5, pages 187-192.	1-56
Y	BIRCHENALL-ROBERTS, ET AL. Inhibition of Murine Monocye Proliferation by a Colonystimulating Factor-1 Antisense Oligonucleotide. The Journal of Immunology. 15 November 1990, Vol. 145, No. 10, pages 3290-6.	1-56
Y	SANSILVESTRI ET AL. Early CD34 high Cells Can Be Separated Into KIThigh Cells in Which Transforming Growth Factor-B (TGF-B) Downmodulates c-kit and KIT low Cells in Which Anti-TGF-B Upmodulates c-kit. Blood. 01 September 1995, Vol. 86, No. 5, pages 1729-1735.	1-56
Y	YOKOYAMA ET AL. Modulation of c-fms proto-oncogene in an ovarian carcinoma cell line by a hammerhead ribozyme. British Journal of Cancer. March 1997, Vol. 76, No. 8, pages 977-982.	1-56